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Antimicrobial and Antioxidant Activities of Four Selected Noncommercial Honeys

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Abstract*—In this study; we investigated the antioxidant activities and antimicrobial effects of some honey samples (Honeydew, Eucalyptus, Petroselinum crispum M. and flower) from different regions of Hatay, Turkey. The antioxidant capacities were evaluated by using folin assay, free radical scavenging (DPPH) assay and β-carotene-linoleic acid emulsion method and the results compared against reference synthetic antioxidants (BHA, BHT). A 96 well minimum inhibitory concentration (MIC) spectrophotometric-based assay is used to assess antimicrobial activity against some gram positive bacteria and gram negative bacteria. Total phenolic content of the honey samples were determined according to the Folin-Ciocalteau procedure and found between 60,58–287,3 mg GAE/kg honey. The characteristic antioxidant activities showed a marked correlation with the total phenolic contents. The honey samples showed low MIC values against three gram (+) and three gram (-) bacteria. Honeydew honey found to be stronger than the other honeys in terms of both antioxidant and antimicrobial potential.*

***Keywords*— Honey, DPPH, total phenolic content, β-carotene assay, MIC.**

I. INTRODUCTION

Natural food usually contains natural antioxidants, which are ubiquitous in fruits, teas, vegetables, cereals, honeys and medicinal plants, can scavenge free radicals. According to the literature natural antioxidants have received great attention and have been studied extensively. The use of natural antioxidants in foods is limited, however, on account of the lack of knowledge concerning their molecular composition, the content of active compounds in the raw material and the availability of relevant toxicological data [1]. Honey has been used since ancient times and has gained appreciation as the only concentrated form of sugar available worldwide [2] and it is known both as food and as a natural product with nutritional, therapeutic and social roles in different cultures. Ancient Egyptians and Greeks used honey as a medicine to treat ailments. Honey has been reported to contain more than 150 substances (complex mixture of sugars and also small amounts of other constituents such as minerals, proteins, vitamins, organic acids, flavonoids, phenolic acids, enzymes and other phytochemicals) and is considered as an important part of traditional medicine [3] and to be effective in the treatment of burns, gastrointestinal disorders, asthma, infected wounds and skin ulcers [4,5]. Many researchers showed that honey serves as a source of natural antioxidants, which are effective in reducing the risk of cardiovascular disease, cancer [6], These diseases are a consequence of oxidative damage and it seems that part of the therapeutic properties of honey is due to its antioxidant capacity. Because of its sweetness, color and flavor, honey is often used as a sugar substitute, an ingredient or a natural preservative in many of manufactured foods and can also prevent oxidation reaction in foods (e.g., lipid oxidation in meat) [7-9] Honeys with dark color have a higher total phenolic content and consequently a higher antioxidant capacity [10]. Honey is generally classified by the floral source of the nectar from which it was made, and there are also divisions according to the packaging and processing used. Previous works have indicated that the antioxidant capacity of honey varies widely, depending on the floral source [11-13,4]. Monofloral honey is made from the nectar of one type of flower. In order to produce monofloral honey, beekeepers keep beehives in an area where the bees have access to only one type of flower. In practice, because of the difficulties in containing bees, a small proportion of any honey will be from additional nectar from other flower types. Some typical European examples include thyme, thistle, heather, acacia, dandelion, sunflower, honeysuckle, and varieties from lime and chestnut trees. In North Africa, such as Egypt, examples include clover, cotton, and citrus, mainly orange blossoms. The main aim of this study was to determine the total phenolic contents, antioxidant levels and antimicrobial activities of several monofloral

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(Honeydew, *Eucalyptus*, *Petroselinum crispum* M. and flower) honey samples from Hatay, Turkey.

II. MATERIALS AND METHODS

Honey samples; Four noncommercial honeys of different floral sources are provided in different areas of Hatay/Turkey. Honeydew honey (secretion honey) is supplied from Serinyol, *Eucalyptus* honey is supplied from Reyhanlı, Petroselinum *crispum M. honey* is supplied from Iskenderun and flower honey is supplied from Antakya/Hatay (Fig.1). All honey samples provided from beekeepers and stored at room temperature in dark until further analysis.

Chemicals; Butylated hydroxyanisole (BHA), Gallic acid, and Folin-Ciocalteu's phenol reagent were purchased from Fluka Chemical Co. (Buchs, Switzerland). β-carotene, butylated hydroxytoluene (BHT), linoleic acid, Tween 40, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma–Aldrich. Anhydrous sodium carbonate (Na_2CO_3), di-Potassium hydrogen phosphate (K_2HPO_4), Potassium dihydrogenphosphate (KH_2PO_4), methanol and chloroform were purchased from Merck. All solvents and chemicals used in the experiments were of analytical grade.

Fig.1: Samples and collected regions

Total phenolic content The Folin–Ciocalteu method was used to determine the total phenolic content (TPC) as reported by Singleton et al. (1999) [14], with some modifications. Briefly each honey sample (1 g) dissolved in methanol (5ml) and filtered through Whatman No: 1 . This solution was used (40 µl) and mixed with 2.4 ml of distilled water and 200 µl non-diluted Folin–Ciocalteu reagents for 3 min and then 0.6 ml of sodium carbonate was added (%20, Na_2CO_3). After incubation in the dark at 25 °C for 2 h, the absorbance of the reaction mixture was measured at 760 nm against a methanol blank using a UV-VIS Spectrophotometer. All measurements were made in triplicate. Gallic acid (0–1000 mg/L) was used as a standard to derive the calibration curve. Total phenolic content was expressed as mg gallic acid equivalents (GAE) per kg of honey.

Determination of DPPH radical scavenging activity; The DPPH radical scavenging activity of honey samples was determined as described by Brand-Williams et al.1995 [15]. Briefly each honey sample (1 g) dissolved in methanol (5ml) and filtered through Whatman No: 1. A 0.1 ml aliquot of each honey sample (12,5–200 mg/ml), BHT and BHA in methanol was added to 2.9 ml of 6×10^{-5} M methanolic solution of DPPH. The mixtures was shaken vigorously and left at 25 °C in the dark for 60 min. The absorbance of the solution was measured at 517 nm, using a Spectrophotometer, against a methanol blank. All measurements were made in triplicate. The radical scavenging activity was expressed as IC_{50} (the concentration of the sample (mg/ml) required to scavenge 50% of DPPH), calculated by a linear regression analysis.

β-carotene bleaching assay;The antioxidant activity of methanolic honey solutions was evaluated by the β-carotene linoleate model system [1]. Briefly, a solution of β-carotene (0,2 mg/ml) was prepared in chloroform and two milliliters of this solution was pipetted into a small (100 ml) round-bottom flask. After removing the chloroform under vacuum at 40 °C; 20 mg of linoleic acid, 200 mg of Tween 40 and 50 ml of distilled water were added to the flask with vigorous shaking. Aliquots (4,8 ml) of the prepared emulsion were transferred to a series of tubes containing 0,2 ml of honey samples. After placing the test tubes in a water bath at 50 °C; the absorbance of each tube steadily was measured using a spectrophotometer at 470 nm by starting zero time absorbance (t=0 min) and at 15-min intervals until the end (t=120 min), of the experiment. BHA and BHT were used as standards. The β-carotene bleaching was calculated using the following equation:

$$Rate\ of\ \beta\text{-}carotene\ bleaching = \ln(A_0/A_t)\, x\ 1/t$$

where A_0 is the initial absorbance of the emulsion at time 0; A_t is the absorbance at 120 min; and t is the time in min. Absorbance of all the sample solutions were measured at 470 nm. The antioxidant activity was described as by the mean percent inhibition of β-carotene bleaching against using the equation:

$$[(R_{control}/R_{sample})/R_{control}]\ x100$$

where $R_{control}$ and R_{sample} are the bleaching rates of β-carotene in the emulsion without antioxidant and with honey samples, respectively.

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Antimicrobial Activity Test

Honey Preparation;vFour different honey types from four different floral origin (Fig. 1) was used and the honey dilution preparations of 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, 5%, 2.5% vol/vol in nutrient broth were prepared and pipetted (100 µL) onto sterile 96-well microtiter plates by using Eppendorf multi channel pipette.

Assay for antimicrobial activity; The antimicrobial activity of the honey samples was studied spectrophotometrically by a modified dilution method for minimum inhibitory concentration (MIC) using three Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 15442) and three Gram-positive bacteria (*Staphylococcus aureus* ATCC 6538, MRSA *Staphylococcus aureus* ATCC 43300, *and Bacillus cereus* ATCC 11778) [16-17]. The tests were performed in sterile 96-well microtiter plates with U – shaped wells. In brief all bacterial strains grown on nutrient agar at 37°C for 24 h and were suspended in Nutrient Broth at density adjusted to a turbidity of 0.5 McFarland standards. The final inoculums were $5x10^5$ CFU/ml of bacterial colony. The wells were filled with 100 µL honey dilutions by using Eppendorf multi channel pipette. After that by using microplate dispenser each well was inoculated with 100 µL of 0.5 McFarland standard bacterial suspensions except the 12^{th} serial of the 96-well microplates. The 11^{th} well were selected as positive control containing medium and microorganisms and the last well were selected as negative control containing only medium. The absorbencies were measured in an ELISA microplate reader at 620 nm prior to incubation, T_0. The plates were lid and incubated at 37 ºC for 24 h. After 24 h, plates were again read in ELISA microplate readerat 620 nm, T_{24}. KCjunier program is used to calculate MIC values which are defined by subtracting the absorbance of each well (T_{24} - T_0) on the value obtained were plotted against the concentrations of wells.

III. RESULTS AND DISCUSSION

The antioxidant properties of different non-commercial honey samples were evaluated using the honey samples and synthetic antioxidant compounds. Numerous antioxidant tests have been developed for food or some biological samples but none of them can be accepted as universal. Therefore generally these methods are modified just as in this study. Higher antioxidant capacity of honeys are depends on their chemical structure especially polyphenolic compounds and flavonoids. These compounds in honey are strongly impressed by their floral origin and climate characteristic of the locations. [18]. Polyphenolic content analyzed of honey samples are introduced in Table 1. Clearly, total phenolic contents in the honey samples are determined as mg gallic acid equivalents (GAE) per kg of honey. According to these results, while honeydew honey sample (287,31 mg.GAE/kg honey) has a rich polyphenolic content than others and phenolic contents of *Eucalyptus* honey (60,58 mg.GAE/kg honey) showed the lowest. The concentration and type of polyphenolic substances in honey is variable and depends on the floral origin of honey [19]. DPPH is a radical and has been widely used to test the free radical scavenging activity of various samples. DPPH free radical gives a strong absorption maximum at 517 nm and is purple in color. Values of IC_{50}, connected to the percent decolorization of DPPH radicals, are shown in Table 1.

Table.1: Antioxidant activity test results of honey samples

	Hh	*Eh*	*Ph*	Fh	BHA	BHT
Phenolic content (mg.GAE/kg honey)	287,3	60,58	121,6	61,71	---	---
IC_{50}	9,85	22,51	16,02	17,62	4,11	5,45
Inhibition %	40,9	5,68	17,04	4,54	27,84	16,47

Hh; Honeydew honey, Eh; *Eucalyptus Honey*, *Petroselinumcrispum M.Honey*,
Fh; Flower Honey

IC_{50} is defined as inhibitory concentration or scavenging effect. The IC_{50} is the concentration of an antioxidant which 50% inhibition of free radical activity is observed. So the lower IC_{50} number indicates higher antioxidant activity [20]. According to the DPPH test results; BHA and BHT are synthetic polyphenolic antioxidant compounds therefore BHA and BHT have lowest IC_{50} value. (4,11 and 5,45 respectively) and this is extremely normal. Among the honey samples, honeydew honey is the lowest than others (IC_{50}: 9,85). Their IC_{50} values of *Petroselinum crispum M*, flower and *Eucalyptus* respectively 16,02, 17,62 and 22,51. The value of correlation coefficient is between -1 and +1. The + and – signs are used for positive linear correlations and negative linear correlations, respectively. The correlation between the free radical scavenging activity and total phenolic

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content was highly statistically significant. The correlation coefficient was equal to (-0.9171) (Table 2).

Table.2: The correlation coefficient values of between antioxidant activity tests.

	Between Folin Method and DPPH assay	Between Folin Method and *β*-carotene bleaching assay.
Correlation coefficient	- 0,9171	+ 0,9970

These results suggest that the antioxidant power of honey samples is especially due to phenolic compounds that are the major constituents having reducing power. For this reason, the coefficient is negative. The antioxidant activity of the honey samples as measured by the bleaching of β-carotene; positive control BHA, BHT, and all samples were able to inhibit the discoloration of β-carotene The order was honeydew honey (40,90%) > BHA (27,84%) > *Petroselinum crispum M.* (17,04%) > BHT (16,47%) > *Eucalyptus* (5,68%) > Flower 4,54%) (Table 1). The correlation coefficient between the inhibition % and total phenolic content was equal to (+0.9970) (Table 2). The antioxidant test results are parallel to each other.

The MIC was determined by the mean of lowest concentration of honey solutions that exhibited the growth of the organisms in the wells by specrophotometric reading accordingly to an Elisa Microplate Reader. Environmental and economical concerns have led analysts towards smaller sample sizes and reduction of the required solvents. However, more realizable results are obtained with larger sample sizes. The MIC results obtained using a broth microdilution method was presented in Table 3.

Table 3. MIC values of honey samples (mg/ml)

	Gram positive			*Gram negative*		
	S.A	*BC*	*MRSA*	*KP*	*EC*	*PA*
Hh	500	500	500	700	500	500
Eh	600	600	600	-	500	400
Fh	500	600	400	800	600	600
Ph	700	600	600	700	600	700

SA; Staphylococcus aureus, BC; Bacillus cereus,KP; Klebsiella pneumoniae
EC; Escherichia coli, PA; Pseudomonas aeruginosa
Hh; Honeydew honey, Eh; *Eucalyptus Honey, Petroselinumcrispum M.Honey,*
Fh; Flower Honey

Honeydew honey found to be stronger than the others in terms of antioxidant power also effective to antimicrobial potential both gram positive and gram negative bacteria. The results showed that honey extracts generally have similar antimicrobial capacity inhibitory, with the exception of the *Klebsiella pneumoniae* microorganisms, but honeydew honey moderately sensitive to the antimicrobial activity. It seems that honey, most effective against *Pseudomonas aeruginosa* is *Eucalyptus* honey. Therewithal the least effective than the others against all bacteria is *Petroselinum crispum M.* honey. Generally all honey samples showed an antioxidant effect with the respect of some differences. These discrepancies could be attributed with the differences of botanical sources of honey and also to the presence of different antioxidant compounds such as some flavonoids, phenolics and phenolic acids [4]. These results show that honey is a natural product with antioxidant and antimicrobial properties. Regarding the antioxidant and antimicrobial properties especially honeydew honey is valuable and its inclusion in the diet may be recommended to complement other polyphenol sources.

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